Cellular Mechanism of the Nonmonotonic Dose Response of Bisphenol A in Rat Cardiac Myocytes

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Abstract

Background: The need for mechanistic understanding of non-monotonic dose responses was identified as one of the major data gaps in the study of bisphenol A (BPA). Previously we reported that acute exposure to BPA promotes arrhythmogenesis in female hearts through alteration of myocyte Ca^{2+} handling, and that the dose response of BPA was inverted-U shaped.

Objective: To define the cellular mechanism underlying the non-monotonic dose response of BPA in the heart.

Methods: Rapid effects of BPA in female rat ventricular myocytes were examined using video-edge detection, confocal and conventional fluorescence imaging, and patch clamp.

Results: The rapid effects of BPA in cardiac myocytes, as measured by multiple endpoints including development of arrhythmic activities, myocyte mechanics and Ca^{2+} transient, were characterized by non-monotonic dose responses. Interestingly, the effects of BPA on individual processes of myocyte Ca^{2+} handling were monotonic. Over the concentration range of 10^{-12} to 10^{-6} M, BPA progressively increased sarcoplasmic reticulum (SR) Ca^{2+} release and Ca^{2+} reuptake and inhibited the L-type Ca^{2+} current (I_{CaL}). These effects on myocyte Ca^{2+} handling were mediated by estrogen receptor (ER) β signaling. The non-monotonic dose responses of BPA can be accounted for by the combined effects of progressively increased SR Ca^{2+} reuptake/release and decreased Ca^{2+} influx through I_{CaL}.

Conclusion: BPA's rapid effects on female rat cardiac myocytes are characterized by non-monotonic dose responses as measured by multiple endpoints. The non-monotonic dose response is produced by ERβ-mediated monotonic effects on multiple cellular Ca^{2+} handling processes. This represents a distinct mechanism underlying the non-monotonicity of BPA's actions.

Introduction

Bisphenol A (BPA; CAS 80-05-7) is one of the highest production volume chemicals worldwide with annual production exceeding 3 million metric tons. It is used in the production of polycarbonate plastics, epoxy resins and non-polymer additives to other plastics. It is used extensively in the manufacturing of common consumer products and goods such as food containers, metal cans (as protective coating), beverage and baby bottles, receipt paper and water pipes (Vandenberg et al. 2007). There is well documented human exposure to BPA from diet, inhalation and other exposure routes (Geens et al. 2012; Vandenberg et al. 2007). BPA was detected in over 90% of individuals in various sample populations (Vandenberg et al. 2010).

BPA is an estrogenic endocrine disrupting chemical (EDC) with potentially adverse impacts on human health (Diamanti-Kandarakis et al. 2009). A large body of evidence has linked BPA exposure to abnormalities such as obesity, diabetes, and disorders of the reproductive and immune systems. Growing evidence also suggests that BPA may have adverse impacts on the cardiovascular system. Epidemiological evidence demonstrates that higher human BPA exposure is associated with cardiovascular diseases including coronary and peripheral arterial diseases (Lang et al. 2008; Melzer et al. 2010; Melzer et al. 2012; Shankar et al. 2012). Recently, we have shown that acute exposure to environmentally relevant low doses of BPA promotes arrhythmogenic triggered activities in cardiac myocytes from female rodent hearts (Belcher et al. 2012; Yan et al. 2011). The pro-arrhythmic action of BPA is manifested as increased frequency of ventricular arrhythmias under stress conditions (Yan et al. 2011), and increased duration and severity of ventricular arrhythmias following ischemic injury in female rodent hearts (Yan et al. 2013). We have demonstrated that alterations of myocyte Ca^{2+} handling, including elevated sarcoplasmic reticulum (SR) Ca^{2+} spontaneous release (or Ca^{2+} leak) and SR Ca^{2+} reuptake, are a

key mechanism underlying the pro-arrhythmic action of BPA (Gao et al. 2013; Yan et al. 2011). These findings point to the potential cardiovascular toxicity of BPA.

Previously we reported that the rapid effect of BPA on the contractility of female cardiac myocytes is characterized by non-monotonic dose response (Belcher et al. 2012). A non-monotonic dose response curve is one that has a point of inflection where the curve slope switches sign from positive to negative or vice versa. Numerous examples of non-monotonic dose response have been reported for a range of EDCs and hormones at the gene expression, cell, tissue/organ, animal, and population levels; see (Vandenberg et al. 2012) for a comprehensive review. Such pharmacodynamic property is of critical importance to the assessment of toxicity of BPA and other EDCs (Fagin 2012; Myers et al. 2009). In the Chapel Hill BPA Statements, the need for investigation of the mechanisms underlying non-monotonic dose responses was identified as one of the major data gaps in the research of BPA (Wetherill et al. 2007). In the present study, we further define the dose response properties of BPA in the heart, and elucidate the underlying mechanism of the non-monotonicity of BPA's cardiac impact.

Methods

Reagents

All reagents and solvents used were of the highest purity available. Aqueous solutions were prepared using BPA-free water (18 MΩ; <6 ppb total oxidizable organics; Millipore A10 system). Bisphenol A (BPA) was from TCI America, lot 111909 (ground by Battelle), and was provided by the Division of the National Toxicology Program (DNTP) at the National Institute of Environmental Health Sciences. Methyl-piperidino-pyrazole,1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP) and 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) were from Tocris Cookson (Ellisville, MO). Nifedipine was from Sigma-Aldrich (St. Louis, MO). Other chemicals were from Sigma-Aldrich unless otherwise stated.

Animals

Animal procedures were done as previously described (Yan et al. 2013), and in accordance with protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee. The animals were treated humanely and with regard for alleviation of suffering. Adult female Sprague-Dawley rats (200-250 grams; Charles River; Spencerville, OH) were housed two per cage under 14-hr light/10-hr dark cycle with lights on at 6AM in standard polycarbonate caging with Sani-chip bedding (Irradiated Aspen Sani-chip; P.J. Murphy Forest Products Corp. Montville, NJ) to eliminate possible corn-based mycoestrogen exposure. All animals are fed *ad libitum* Teklad diet 2020 (Harlan Laboratories Inc.) which lacks soybean meal, alfalfa or animal products that may introduce uncontrolled levels of estrogenic compounds. Sterile drinking water was generated by a dedicated water purification system (Millipore Rios 16 with ELIX UV/Progard 2) that reduces oxidizable organics to less than 1% of source levels.

Drinking water was dispensed from glass water bottles. A total of 70 animals were used in this study.

Analysis of myocyte mechanics and Ca^{2+} handling

Ventricular myocytes from female rat hearts were enzymatically dissociated using Langendorff perfusion as previously described (Yan et al. 2011; Yan et al. 2013). Isolated myocytes were suspended in 1.0 mM Ca^{2+}-Tyrode solution for immediate experiments. Analysis of myocyte contraction, after-contraction and Ca^{2+} transient and spark were performed as previously described (Yan et al. 2011). Briefly, myocytes were excited with field stimulation with 2 msec 1.5x threshold pulses at a rate of 0.5 Hz. Steady-state myocyte shortening was examined using a video-edge detector (Crescent Electronics, Sandy, UT). After-contraction was measured with stimulation of 2 msec 1.5x threshold pulses at a rate of 2 Hz for 8 sec. To measure Ca^{2+} spark, isolated ventricular myocytes were loaded with fluo-4 acetoxymethyl ester (5 μM; Molecular Probes, Eugene, OR) and imaged with a Zeiss LSM 710 inverted confocal microscope (Carl Zeiss Microscopy, LLC, Model Zeiss-LSM710, Thornwood, NY) with excitation wavelength of 488 nm. Signals were measured with line-scan imaging at 3.07 ms intervals, with each line comprising 512 pixels spaced at 0.056 mm. Image processing and data analysis were performed as previously described (Yan et al. 2011). To measure Ca^{2+} transients, fluorescence signals were measured from fluo-4 loaded myocytes using a Nikon TE 2000 microscope and an InCyt Standard PM photometry system (Intracellular Imaging, Cincinnati, OH). Experiments were performed at room temperature (24°C).

Patch clamp recording of I_{CaL}

I_{CaL} was recorded at room temperature (24 °C) using the whole-cell patch clamp technique as previously described (Yan et al. 2011). After the membrane was ruptured, cells were clamped at –50 mV for 5 minutes to allow dialysis of the intracellular solution and stabilization of the Ca^{2+} currents before measurement began. Data collection and analysis were performed using PCLAMP 9 software.

Western blotting

Western blotting experiments were performed as previously described (Gao et al. 2013). Briefly, isolated female ventricular myocytes were treated with BPA for the indicated length of time, collected and snap-frozen in liquid nitrogen. Proteins were extracted with 1x Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitors and phosphatase inhibitors. Equal amounts of protein samples from each treatment group were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then blocked with 5% non-fat milk in PBS - 0.1% Tween, following incubation with primary and secondary antibodies. ECL™ Western Blotting Analysis System (GE Healthcare, UK) was used for developing the membrane. Antibodies used in this study were pThr17-PLN, PLN (Badrilla, UK), horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Cell Signaling Technology, Danvers, MA).

Statistical analysis

Statistical analysis was conducted using unpaired t-test, or one-way analysis of variance (ANOVA) with differences between treatment groups assessed using a multiple comparison post-test. Frequency of events (e.g., percentage of myocyte with after-contractions) was analyzed

using a chi-square (χ^2) test. Minimal level of statistical significance for differences in values is considered to be $P < 0.05$. Data was analyzed with SigmaPlot and Excel.

Results

Rapid actions of BPA in cardiac myocytes have non-monotonic dose responses

We examined the concentration-dependent effect of BPA on Ca^{2+} transient in female rat ventricular myocytes. BPA rapidly (~ 5 minutes) increased the amplitude of the Ca^{2+} transient at low doses (10^{-10} to 10^{-8} M), and this stimulatory effect diminished at micromolar dose (Figure 1A1); the dose response curve was inverted-U shaped with a most efficacious concentration of 10^{-8} M (Figure 1A2). Previously we showed that BPA rapidly promoted the development of spontaneous excitation (i.e., triggered activities) in female cardiac myocytes (Yan et al. 2011). The stimulatory effect of BPA on triggered activities was only notable in the nanomolar dose range but not at higher doses (Figure 1B1), producing a dose response curve with pronounced non-monotonicity (Figure 1B2).

One mechanism of the non-monotonic dose response of hormones and EDCs is general cytotoxicity (Vandenberg et al. 2012); this possibility was examined. Consistent with our previous findings (Belcher et al. 2012), 10^{-8} M BPA rapidly stimulated the contraction of female myocytes and this stimulatory effect diminished at micromolar concentration (Figure 1C1). Myocytes were pre-treated with 10^{-6} M BPA followed by wash; these cells responded robustly to subsequent exposure to 10^{-8} M BPA (Figure 1C2). In addition, the β-adrenergic agonist isoproterenol produced marked stimulatory effect in the presence of 10^{-6} M BPA. These results suggest that the diminished response of myocytes to micromolar BPA is not due to non-specific cytotoxicity of BPA at higher dose.

BPA alters individual elements of myocyte Ca^{2+} handling with monotonic dose responses

BPA promotes arrhythmogenesis and enhances myocyte contraction via alteration of myocyte Ca^{2+} handling (Yan et al. 2011). To understand the mechanism of the non-monotonicity of BPA's rapid actions in cardiac cells, we examined the dose-dependent impact of BPA on the individual elements of the myocyte Ca^{2+} handling process.

Effect on Ca^{2+} spark

Increased Ca^{2+} release and Ca^{2+} leak from the SR play a central role in the impact of BPA on arrhythmogenesis and myocyte mechanics (Yan et al. 2011). Diastolic Ca^{2+} release from the SR through the ryanodine receptors was measured as frequency of Ca^{2+} sparks (Cheng et al. 1993). Interestingly, unlike the non-monotonic dose responses observed at the myocyte level, BPA's impact on SR Ca^{2+} release was monotonic (Figure 2). Increasing concentrations of BPA over the dose range of 10^{-10} to 10^{-5} M progressively increased Ca^{2+} spark frequency (Figure 2A and 2B). The dose response curve had a classic monotonic shape with an EC_{50} of 0.81 nM and a maximum increase of 209.7% (Figure 2C).

Effect on SR Ca^{2+} reuptake

BPA rapidly increases SR Ca^{2+} reuptake in female rat myocytes (Yan et al. 2011). In rodent cardiac myocytes, SR reuptake accounts for most (> 90%) of Ca^{2+} removal from the cytosol during relaxation (Bers 2002); therefore, the rate of decline of the Ca^{2+} transients can be used as an index of SR Ca^{2+} reuptake. Whereas the effect of BPA on Ca^{2+} transient amplitude was inverted-U shaped as discussed earlier (Figure 3A, upper panel), normalization of the Ca^{2+} transient traces revealed that increasing concentrations of BPA progressively increased the rate

of decline (Figure 3A, lower panel), producing a monotonic dose response curve (Figure 3B). The dose response had an EC_{50} of 0.15 nM and a maximum increase of 37.3% (Figure 3C).

Through its inhibition of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), phospholamban (PLN) is the central regulator of SR Ca^{2+} reuptake. PLN can be phosphorylated by both PKA and CAMKII, at serine 16 and threonine 17 respectively. Phosphorylation of PLN releases its inhibition on SERCA, thereby increasing Ca^{2+} reuptake into the SR (Kranias and Hajjar 2012). Previously, we showed that BPA influences SR Ca^{2+} reuptake via increasing CAMKII phosphorylation of PLN at threonine 17 (Gao et al. 2013). The dose-dependent effect of BPA (2 minute exposure) on CAMKII phosphorylation of PLN was examined. Increasing concentrations of BPA over the dose range of 10^{-12} M to 10^{-6} M progressively increased phosphorylation of PLN at the CAMKII site (Figure 3D). Based on known regulatory mechanism of SR Ca^{2+} reuptake, such dose-dependent effect of BPA on PLN phosphorylation should increase SERCA activity and likely accounts for the monotonic effect of BPA on SR Ca^{2+} reuptake.

Effect on I_{CaL}

The stimulatory, monotonic effects of BPA on SR Ca^{2+} release and reuptake are countered by an inhibitory action of BPA on the L-type Ca^{2+} current, particularly at higher concentrations (Figure 4A). The dose response curve for the inhibition of I_{CaL} was, again, monotonic (Figure 4B), with an EC_{50} of 27.4 nM and a maximum inhibition of 43%. The current-voltage relationship of I_{CaL} was not changed by BPA.

Interestingly, although the overall effect of BPA on I_{CaL} was one of suppression, at lower concentrations (10^{-12} M) a small but consistent stimulation of the current was noticed (Figure

4C). The average increase of I_{CaL} at 10^{-12} M BPA was 4.5%, which occurred rapidly within minutes of exposure.

BPA's rapid actions are mediated by ERβ signaling

Previously we hypothesized that the opposing actions of ERα and ERβ contribute to the non-monotonic dose response of BPA in the heart (Belcher et al. 2012). Contrary to this hypothesis, in the presence of MPP, an ERα selective blocker, the non-monotonicity of the dose response curve was unchanged (Figure 5A); ERβ blockade with PHTPP largely abolished the rapid effect of BPA on contractility (Figure 5B).

We also examined the role of ERβ in mediating BPA's effects on individual Ca^{2+} cycling processes. ERβ blockade with PHTPP completely abolished the stimulatory effect of BPA on Ca^{2+} spark frequency (Figure 5, C1 and C2), and blocked effects of BPA on the amplitude and decline kinetics of Ca^{2+} transient (Figure 5, D1 and D2). Similarly, PHTPP abolished the inhibitory effect of BPA (10^{-5} M) on I_{CaL} (Figure 5, E1 and E2).

Thus, ERβ signaling plays a dominant role in mediating BPA's non-monotonic dose response in cardiac myocytes as well as its actions on individual Ca^{2+} cycling processes.

Suppression of I_{CaL} contributes to the non-monotonic effects of BPA

We tested the hypothesis that the inhibitory effect of high dose BPA on I_{CaL} produces the non-monotonic dose responses, using the selective I_{CaL} blocker nifedipine to mimic the inhibition by BPA. At 10^{-8} M, BPA's stimulatory effects on SR Ca^{2+} release and reuptake are near or at saturation (Figures 2 and 3), while it only inhibits I_{CaL} by 11% (Figure 4). 0.2 μM nifedipine was used in our experiments; at this dose, nifedipine blocked I_{CaL} by 39% (Figure 6, A1 and A2),

which is consistent with the reported IC_{50} value of nifedipine of 0.3 μM (Shen et al. 2000). The dose response of BPA, over the dose range of 10^{-12} to 10^{-8} M, plus 10^{-8} M BPA combined with 0.2 μM nifedipine were tested. Addition of nifedipine to 10^{-8} M BPA reproduced the inverted-U shaped dose responses as measured by both myocyte contractility (Figure 6, B1 and B2) and incidence of triggered activities (Figure 6, C1 and C2). Thus, the monotonic and stimulatory effects of BPA on SR Ca^{2+} release and reuptake, plus the inhibitory effect of higher dose BPA on I_{CaL}, are sufficient to produce the non-monotonic dose responses of BPA in female rat cardiac myocytes.

Discussion

Defining the non-monotonic dose responses of EDCs is of both scientific interest and relevance to understanding the potential health impacts of these chemicals. Here, we show that acute exposure to low doses of BPA (at or below 10^{-8} M) has significant impact on arrhythmogenesis and mechanics of female cardiac myocytes, and that the dose responses of the rapid impact of BPA are non-monotonic. The non-monotonicity is produced by multiple monotonic effects on individual cellular Ca^{2+} handling processes through ERβ-mediated signaling (Figure 6D). As a case study, our results add to a growing body of evidence demonstrating the non-monotonic responses of EDCs, and provide mechanistic insights into the pharmacodynamics of BPA's actions.

Non-monotonic dose responses of EDCs and hormones have been well documented and are likely a common phenomenon of EDCs (Vandenberg et al. 2012). Nevertheless, their existence and significance have been disputed (Rhomberg and Goodman 2012), citing the lack of statistical significance in some studies and attributing the observations to random dose-by-dose

fluctuations and high signal-to-noise ratio. In addition, existing evidence of non-monotonicity has been faulted for involving only quantitative continuous endpoints and lacking all-or-none biological events. The lack of mechanistic understanding of the link between quantitatively continuous effects of EDCs and changes in incidence rates of distinct diseases is viewed as a weakness. We assessed the dose response of BPA in cardiac cells using multiple endpoints including myocyte contraction, Ca^{2+} dynamics and arrhythmogenesis, using separate and independent measurement assays; each produced dose responses with marked non-monotonicity. The response measured at 10^{-6} M for each of the endpoint showed clear and statistically significant ($P < 0.01$) declines compared with that at 10^{-8} M, and had no statistical difference ($P > 0.2$) compared with control (Figure 1). Given the consistency among multiple endpoints, statistical significance of the results, and reproducibility of these non-monotonic events from previous studies (Yan et al. 2011), it is considered unlikely that the observed non-monotonic dose responses are attributable to random fluctuation. Of particular significance is the arrhythmogenesis endpoint (i.e., incidence of triggered activity). Triggered activities are aberrant spontaneous excitations of cardiac myocytes and are well-recognized as one of the key arrhythmogenic mechanisms in the heart (Pogwizd and Bers 2004); as such, they could be considered a toxicologically relevant endpoint. The presence or absence of triggered activity is clearly an all-or-none event and was measured as such in our study. The pronounced inverted-U shape of BPA's effect on this all-or-none endpoint is evident. Other examples of BPA impacting all-or-none events with non-monotonic dose response include the presence/absence of tumor and metastases [e.g., (Jenkins et al. 2011)]. Thus, the non-monotonic dose response of BPA is not limited to quantitative continuous endpoints.

Growing evidence has provided increasing understanding of the mechanisms that generate the non-monotonic dose response of EDCs. The known mechanisms are diverse, and have been reviewed in a number of articles (Myers et al. 2009; Vandenberg et al. 2012; Watson et al. 2010). Examples of these mechanisms include actions of different types of receptors (e.g., ERα and ERβ) with opposing signaling effects, cell subpopulation-specific and opposite responses to hormone actions, cytotoxicity associated with higher hormone doses resulting in decreased responses, signaling through parallel pathways with different temporal activation patterns, receptor desensitization, and receptor down-regulation at higher doses. It should also be noted that a range of other xenobiotics are capable of producing non-monotonic effects due to non-specific mechanisms of action. Here, we describe a distinct mechanism in cardiac myocytes that involves signaling of a single receptor, ERβ, that results in multiple monotonic effects on individual elements of the myocyte Ca^{2+} handling process (Figure 6D). Previously, we showed that the rapid effect of BPA on cardiac arrhythmogenesis and mechanics is mediated by its impact on myocyte Ca^{2+} handling; In particular, increased diastolic SR leak plays a key role in the arrhythmogenic effect of BPA (Yan et al. 2011). Our current results demonstrate that BPA rapidly increases SR Ca^{2+} release/leak and SR reuptake with monotonic dose responses. Opposing this stimulatory effect is the monotonic suppression of I_{CaL} at micromolar doses. Based on known mechanisms of cardiac Ca^{2+} handling, suppression of I_{CaL} also reduces Ca^{2+}-induced Ca^{2+} release from the SR, resulting in reduced Ca^{2+} transient amplitude and myocyte contraction. Suppression of Ca^{2+} influx through I_{CaL} may reduce intracellular Ca^{2+}, thereby reducing the development of triggered activities, particularly delayed after depolarizations (Pogwizd and Bers 2004). The exact impact of I_{CaL} inhibition in the presence of enhanced SR Ca^{2+} cycling is complex and influenced by feedback regulatory mechanisms (Eisner et al. 2013). To test role of

I_{CaL} suppression in generating the decline phase of the inverted-U shaped dose response curve, we show that mimicking BPA's suppression of I_{CaL} with the Ca^{2+} channel blocker nifedipine, at a dose that produces a percentage blockade of I_{CaL} similar to that by high dose BPA, reproduced the inverted-U shaped curve. We do recognize that the result of this experiment is confounded by the fact that 10^{-8} M BPA, which does produce a 10% blockade of I_{CaL}, plus nifedipine were used to mimic the effect of micromolar BPA, and this overlapping effects of BPA and nifedipine on I_{CaL} do not fully reproduce the effect of micromolar BPA. Nevertheless, the result qualitatively demonstrates that the non-monotonic effect of BPA can be accounted in part by I_{CaL} inhibitory.

Suppression of the L-type Ca^{2+} channel by high dose BPA shares much similarity with the suppression by high concentrations of 17β-estradiol (E_2), suggesting the possibility of a common mechanism. A number of studies have shown that supra-physiological E_2 (10 to 30 μM) partially suppresses I_{CaL} in cardiac myocytes in multiple species including rat, human and guinea pig (Berger et al. 1997; Kurokawa et al. 2008; Meyer et al. 1998; Nakajima et al. 1999). E_2 has been shown to suppress I_{CaL} in guinea pig ventricular myocytes with a IC_{50} of 29.5 nM and a maximum suppression of 40% (Kurokawa et al. 2008), values that are remarkably similar to the dose response for the blockade by BPA. Such suppression of I_{CaL} by high doses of estrogens, while of limited physiological relevance, may play a role in determining the dose response properties of other estrogenic chemicals in the heart.

It has been shown both experimentally and computationally that non-monotonic dose responses can be generated by the opposing actions of multiple receptors (Conolly and Lutz 2004; Vandenberg et al. 2012; Watson et al. 2010). In previous studies we showed that ERα and ERβ have opposing actions in cardiac myocytes (ERα rapid signaling has an inhibitory effect while

ERβ is stimulatory); thus, the rapid actions of BPA in female hearts are mediated by the stimulatory signaling of ERβ, and the counterbalance of ERα vs ERβ results in the lack of observable response in male hearts to BPA (Belcher et al. 2012). Indeed, we speculated previously that the opposing actions of ERα and ERβ generate the non-monotonic dose response of BPA in the heart (Belcher et al. 2012). This, however, does not appear to be the case. As shown in Figure 5, pharmacological blockade of ERβ largely abolished the rapid effect of BPA on contractility, whereas blockade of ERα has no detectable effect on the inverted-U shaped dose response. Although the sum of BPA's effects on multiple Ca^{2+} handling processes is sufficient to account for the observed non-monotonicity, the potential contribution of other mechanisms, such as receptor desensitization, have not been examined in the present study and cannot be ruled out. In addition, the molecular/signaling mechanisms underlying BPA's impact on the Ca^{2+} handling elements are unknown and remain to be elucidated.

In summary, BPA's rapid effects on female rat cardiac myocytes are characterized by non-monotonic dose responses as measured by multiple endpoints; the cellular mechanism of BPA's non-monotonicity involves monotonic and opposing effects on multiple Ca^{2+} handling processes, all mediated by ERβ signaling. The summation of these parallel effects generates an inverted-U shaped dose response with the most efficacious dose around 10^{-8} to 10^{-9} M, coinciding with the reported human exposure levels (Vandenberg et al. 2010) (Figure 6D).

References

Belcher SM, Chen Y, Yan S, Wang H-S. 2012. Rapid estrogen receptor-mediated mechanisms determine the sexually dimorphic sensitivity of ventricular myocytes to 17beta-estradiol and the environmental endocrine disruptor bisphenol a. Endocrinology 153:712-720.

Berger F, Borchard U, Hafner D, Putz I, Weis TM. 1997. Effects of 17beta-estradiol on action potentials and ionic currents in male rat ventricular myocytes. Naunyn Schmiedebergs Arch Pharmacol 356:788-796.

Bers DM. 2002. Cardiac excitation-contraction coupling. Nature 415:198-205.

Cheng H, Lederer WJ, Cannell MB. 1993. Calcium sparks: Elementary events underlying excitation-contraction coupling in heart muscle. Science 262:740-744.

Conolly RB, Lutz WK. 2004. Nonmonotonic dose-response relationships: Mechanistic basis, kinetic modeling, and implications for risk assessment. Toxicol Sci 77:151-157.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. 2009. Endocrine-disrupting chemicals: An endocrine society scientific statement. Endocr Rev 30:293-342.

Eisner D, Bode E, Venetucci L, Trafford A. 2013. Calcium flux balance in the heart. J Mol Cell Cardiol 58:110-117.

Fagin D. 2012. Toxicology: The learning curve. Nature 490:462-465.

Gao X, Liang Q, Chen Y, Wang H-S. 2013. Molecular mechanisms underlying the rapid arrhythmogenic action of bisphenol a in female rat hearts. Endocrinology 154:4607-4617.

Geens T, Aerts D, Berthot C, Bourguignon JP, Goeyens L, Lecomte P, et al. 2012. A review of dietary and non-dietary exposure to bisphenol-a. Food Chem Toxicol 50:3725-3740.

Jenkins S, Wang J, Eltoum I, Desmond R, Lamartiniere CA. 2011. Chronic oral exposure to bisphenol a results in a nonmonotonic dose response in mammary carcinogenesis and metastasis in mmtv-erbb2 mice. Environ Health Perspect 119:1604-1609.

Kranias EG, Hajjar RJ. 2012. Modulation of cardiac contractility by the phospholamban/serca2a regulatome. Circ Res 110:1646-1660.

Kurokawa J, Tamagawa M, Harada N, Honda S, Bai CX, Nakaya H, et al. 2008. Acute effects of oestrogen on the guinea pig and human ikr channels and drug-induced prolongation of cardiac repolarization. J Physiol 586:2961-2973.

Lang IA, Galloway TS, Scarlett A, Henley WE, Depledge M, Wallace RB, et al. 2008. Association of urinary bisphenol a concentration with medical disorders and laboratory abnormalities in adults. JAMA 300:1303-1310.

Melzer D, Rice NE, Lewis C, Henley WE, Galloway TS. 2010. Association of urinary bisphenol a concentration with heart disease: Evidence from nhanes 2003/06. PLoS One 5:e8673.

Melzer D, Osborne NJ, Henley WE, Cipelli R, Young A, Money C, et al. 2012. Urinary bisphenol a concentration and risk of future coronary artery disease in apparently healthy men and women. Circulation 125:1482-1490.

Meyer R, Linz KW, Surges R, Meinardus S, Vees J, Hoffmann A, et al. 1998. Rapid modulation of l-type calcium current by acutely applied oestrogens in isolated cardiac myocytes from human, guinea-pig and rat. Exp Physiol 83:305-321.

Myers JP, Zoeller RT, vom Saal FS. 2009. A clash of old and new scientific concepts in toxicity, with important implications for public health. Environ Health Perspect 117:1652-1655.

Nakajima T, Iwasawa K, Oonuma H, Morita T, Goto A, Wang Y, et al. 1999. Antiarrhythmic effect and its underlying ionic mechanism of 17beta-estradiol in cardiac myocytes. Br J Pharmacol 127:429-440.

Pogwizd SM, Bers DM. 2004. Cellular basis of triggered arrhythmias in heart failure. Trends Cardiovasc Med 14:61-66.

Rhomberg LR, Goodman JE. 2012. Low-dose effects and nonmonotonic dose-responses of endocrine disrupting chemicals: Has the case been made? Regul Toxicol Pharmacol 64:130-133.

Shankar A, Teppala S, Sabanayagam C. 2012. Bisphenol a and peripheral arterial disease: Results from the nhanes. Environ Health Perspect 120:1297-1300.

Shen JB, Jiang B, Pappano AJ. 2000. Comparison of l-type calcium channel blockade by nifedipine and/or cadmium in guinea pig ventricular myocytes. J Pharmacol Exp Ther 294:562-570.

Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. 2007. Human exposure to bisphenol a (bpa). Reprod Toxicol 24:139-177.

Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol a. Environ Health Perspect 118:1055-1070.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Jr., Lee DH, et al. 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr Rev 33:378-455.

Watson CS, Jeng YJ, Kochukov MY. 2010. Nongenomic signaling pathways of estrogen toxicity. Toxicol Sci 115:1-11.

Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, et al. 2007. In vitro molecular mechanisms of bisphenol a action. Reprod Toxicol 24:178-198.

Yan S, Chen Y, Dong M, Song W, Belcher SM, Wang H-S. 2011. Bisphenol a and 17β-estradiol promote arrhythmia in the female heart via alteration of calcium handling PLoS One 6:e25455.

Yan S, Song W, Chen Y, Hong K, Rubinstein J, Wang H-S. 2013. Low-dose bisphenol a and estrogen increase ventricular arrhythmias following ischemia-reperfusion in female rat hearts. Food Chem Toxicol 56C:75-80.

Figure Legends

Figure 1. Non-monotonic dose responses of BPA in female rat ventricular myocytes. (*A1*) Representative Ca^{2+} transient traces upon acute exposure to indicated concentrations of BPA or control solution. (*A2*) Dose response of the effect of BPA on myocyte Ca^{2+} transient amplitude; n = 23, 18, 17 and 17 myocytes. (*B1*) Representative confocal images of Ca^{2+} transients in female rat myocytes elicited by repeated pacing, under control and upon acute exposure to indicated concentrations of BPA. Arrows indicate spontaneous Ca^{2+} after-transients (i.e., triggered activity) following pacing. (*B2*) Dose response of the effect of BPA on the percentage of myocytes with triggered activity; n = 40, 40, 41, 43 and 41 myocytes. (*C1*) Representative contraction traces of myocytes exposed to indicated treatments (2-7 minute exposures). Iso: isoproterenol. FS: fractional shortening. (*C2*) Average fractional shortening under indicated treatments; n = 20, 19, 20, 20, 20 and 20 myocytes. Error bars are SEM. *: P<0.05; **: P<0.01; ***: P<0.001 vs. control in a one-way ANOVA or a χ^2 test. †: P < 0.01 in an unpaired t-test.

Figure 2. BPA increases SR Ca^{2+} release/leak in female rat ventricular myocytes with a monotonic dose response. (*A*) Ca^{2+} sparks recorded from three representative myocytes, before and after exposure (1 – 2 minutes) to indicated concentrations of BPA. Spot-shaped fluorescent signals indicated local elevations of intracellular Ca^{2+} levels as a result of spontaneous release of Ca^{2+} from the SR (i.e., Ca^{2+} sparks). (*B*) Average spark frequency under control and BPA; n = 8, 7, 17 and 11 myocytes. (*C*) Dose response curve of BPA's effect on Ca^{2+} spark frequency. Data are fitted with the Hill equation: % increase = max increase/$(1 + (EC_{50}/[BPA])^{\text{Hill coefficient}})$, where max increase = 209.7%, EC_{50} = 0.814 nM, and Hill coefficient = 0.34. Error bars are SEM. **: P<0.01; ***: P<0.001 vs. control in a paired t-test.

Figure 3. BPA increases SR Ca^{2+} reuptake in female rat ventricular myocytes with a monotonic dose response. (*A*) Top panel, representative Ca^{2+} transient traces upon acute exposure to indicated concentrations of BPA or control solution. Bottom panel, the same traces normalized to an amplitude (F/F_0) of 1; superimposed red lines are the single exponential fitting of the decline phase of the Ca^{2+} transient in control. Rate of Ca^{2+} transient decline (i.e., 1/time constant) indicated SR Ca^{2+} reuptake in rodent cardiac myocytes. (*B*) Average rate of decline (normalized to control) under control and various concentrations of BPA; n = 23, 18, 17 and 17 myocytes. (*C*) Dose response curve of BPA's effect on Ca^{2+} transient decline rate. Data are fitted with the Hill equation: % increase = max increase/(1 + EC_{50}/[BPA]), where max increase = 37% and EC_{50} = 0.152 nM. *(D)* Rapid impact of BPA on PLN phosphorylation by CAMKII in female rat myocytes. Shown are representative immunoblot of PLN threonine 17 (CAMKII site) phosphorylation and total PLN under control and upon exposure to indicated concentration of BPA for 2 minutes. Error bars are SEM. *: $P<0.05$; **: $P<0.01$ vs. control in a one-way ANOVA.

Figure 4. BPA inhibits the L-type Ca^{2+} current in female rat ventricular myocytes with a monotonic dose response. (*A*) Representative I_{CaL} recorded from the same myocyte under control conditions and upon exposure to various concentrations of BPA. Inset: voltage clamp protocol. (*B*) Dose response of the inhibition of I_{CaL} by BPA; n = 7, 5, 7, 4. Data are fitted with the Hill equation: % inhibition = max inhibition/(1 + IC_{50}/[BPA]), where max inhibition = 43% and IC_{50} = 27.4 nM. Error bars are SEM. (*C*) Example of a small but reproducible increase of I_{CaL} upon exposure to 10^{-12} M BPA. Top panel shows I_{CaL} recorded at 0 mV; bottom panel shows the time course of the observed stimulatory effect. All currents were recorded at steady-state following BPA treatment.

Figure 5. ERβ signaling mediates the rapid effects of BPA in female rat myocytes. *(A)* and *(B)* Effects of MPP and PHTPP, respectively, on the dose response of BPA on myocyte contractility. N = 39, 39, 41, 44 and 40 for (*A*) and 24, 24, 21, 24 and 22 for (*B*). (*C1*) Ca^{2+} sparks recorded from a representative myocyte, before and after exposure (1 – 2 minutes) to BPA plus PHTPP. (*C2*) Average spark frequency under control, BPA and BPA plus PHTPP; n = 9, 4 and 5 myocytes. (*D1*) Representative Ca^{2+} transient traces in control and BPA plus PHTPP. (*D2*) Average Ca^{2+} transient amplitude (left) and decline rate (right) in control, BPA and BPA plus PHTPP; n = 7, 9 and 9 myocytes. (*E1*) Representative I_{CaL} recorded under control and BPA plus PHTPP. Inset: voltage clamp protocol. (*E2*) Average inhibition of I_{CaL} by BPA (10^{-5}) and BPA plus PHTPP; n = 6 and 3, respectively. Error bars are SEM. #: P>0.1; *: P<0.05; **: P<0.01; ***: P<0.001 vs. control in a one-way ANOVA. PHTPP = 5 x 10^{-6} M for all experiments.

Figure 6. Suppression of the L-type Ca^{2+} current reproduces the non-monotonic effects of BPA. (*A1*) Current traces and (*A2*) current-voltage relationship showing the blockade of I_{CaL} by 2 x 10^{-7} M nifedipine. Inset: voltage clamp protocol. (*B1*) Representative contraction traces under control, BPA (10^{-8} M), and BPA (10^{-8} M) plus nifedipine (Nif; 2 x 10^{-7} M). FS: fractional shortening. (*B2*) Dose-dependent effects of BPA (10^{-12} to 10^{-8} M), and effect of BPA (10^{-8} M) plus nifedipine (Nif) on myocyte fractional shortening; n = 35, 23, 23, 35, 37 myocytes. (*C1*) Representative confocal images of Ca^{2+} transients in female rat myocytes elicited by repeated pacing, under control and upon acute exposure to BPA, and BPA plus nifedipine. Arrow indicates triggered activity following pacing. (*C2*) Dose-dependent effects of BPA, and effect of BPA plus nifedipine on incidence of triggered activity; n = 31, 32, 32, 33, 31 myocytes. (*D*) Schematic illustration of the cellular mechanism of the non-monotonic dose response of BPA in female rat ventricular myocytes.

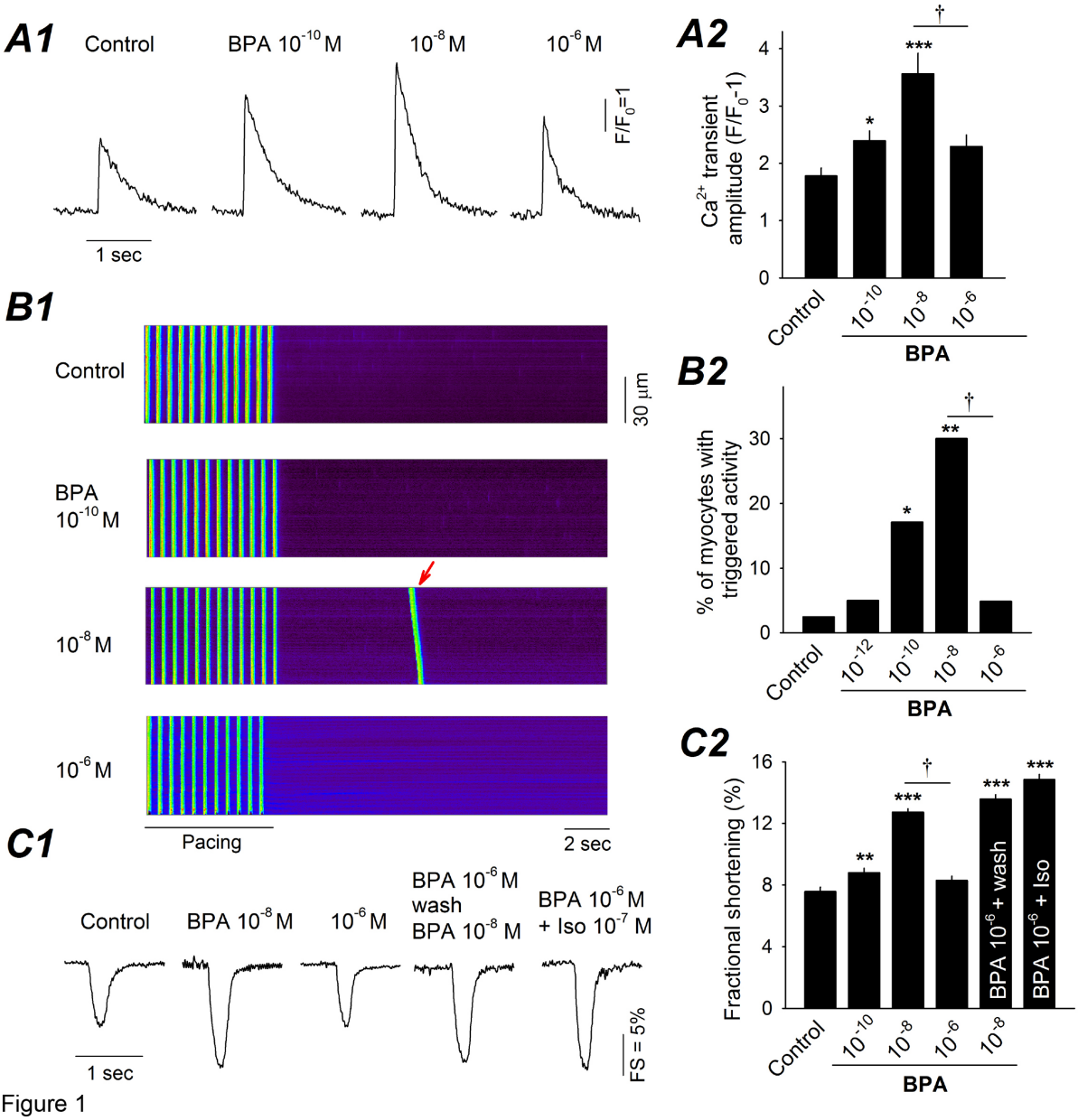
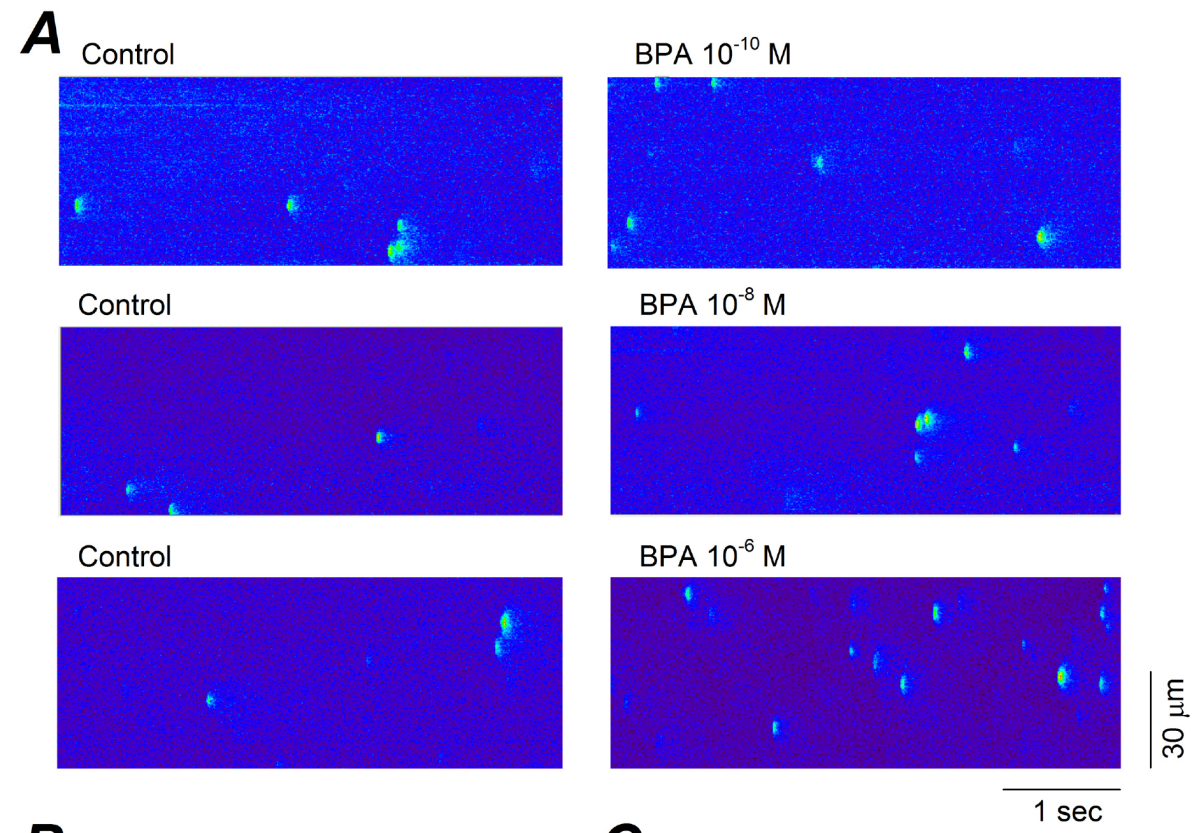
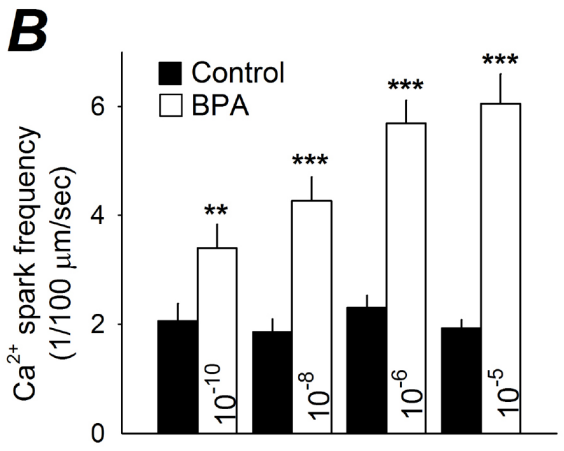

A1
Control
BPA 10-10 M
10-8 M
10-6 M
F/F0=1
1 sec
A2
Ca2+ transient amplitude (F/F0-1)
Control
10-10
10-8
10-6
BPA
B1
Control
30 µm
BPA 10-10 M
10-8 M
10-6 M
Pacing
2 sec
B2
% of myocytes with triggered activity
Control
10-12
10-10
10-8
10-6
BPA
C1
Control
BPA 10-8 M
10-6 M
BPA 10-6 M wash BPA 10-8 M
BPA 10-6 M + Iso 10-7 M
1 sec
FS = 5%
C2
Fractional shortening (%)
BPA 10-6 + wash
BPA 10-6 + Iso
Control
10-10
10-8
10-6
10-8
BPA

Figure 1


A
Control
BPA 10-10 M
Control
BPA 10-8 M
Control
BPA 10-6 M
30 µm
1 sec


B
Control
BPA
Ca2+ spark frequency (1/100 µm/sec)
0
2
4
6
**
***
***
***
10-10
10-8
10-6
10-5

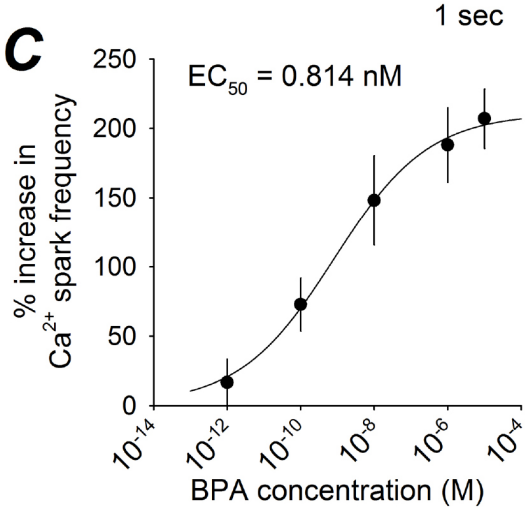

C
EC50 = 0.814 nM
% increase in Ca2+ spark frequency
0
50
100
150
200
250
10-14
10-12
10-10
10-8
10-6
10-4
BPA concentration (M)

Figure 2

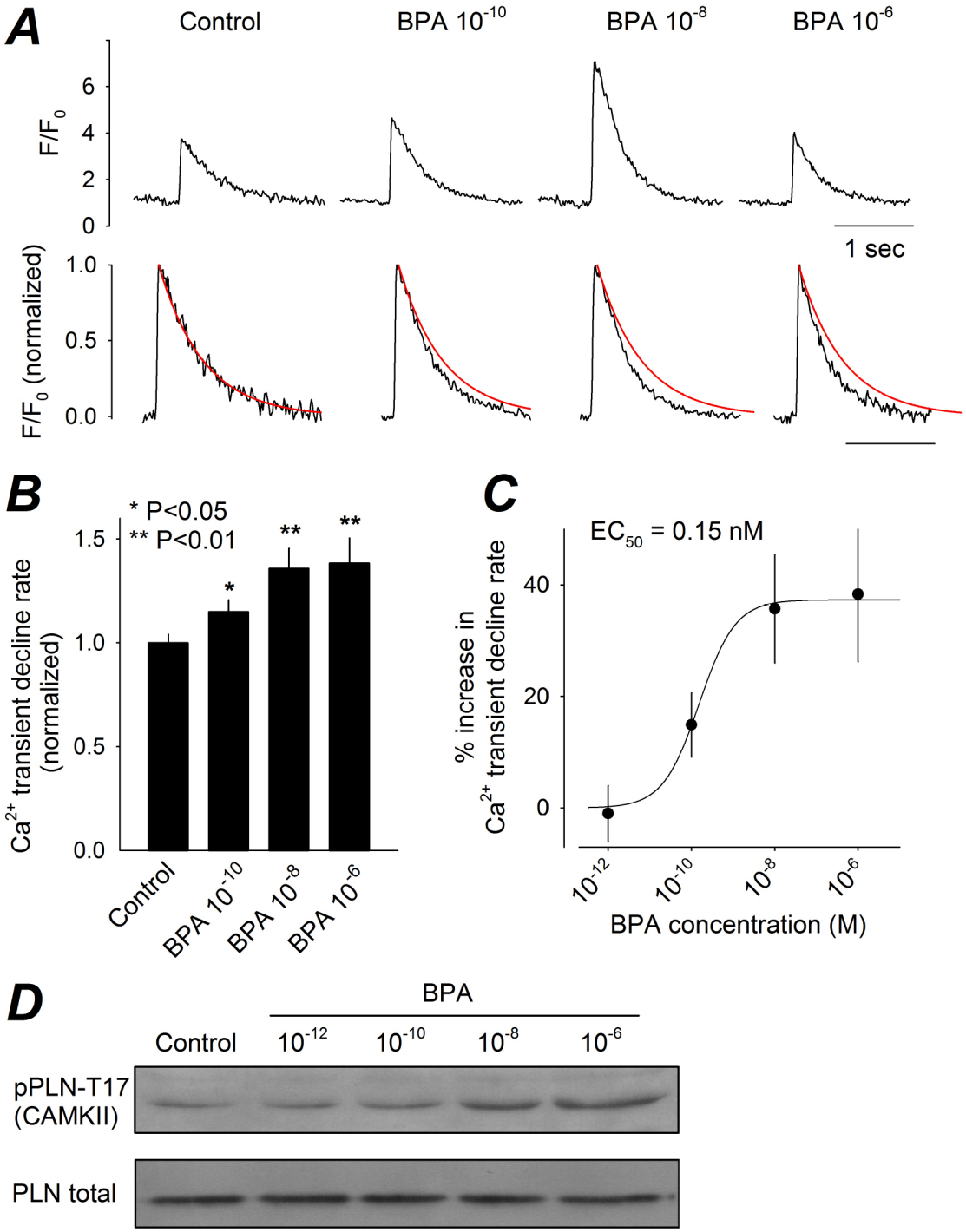

A
Control
BPA 10-10
BPA 10-8
BPA 10-6
F/F0
1 sec
F/F0 (normalized)
B
* P<0.05
** P<0.01
Ca2+ transient decline rate (normalized)
Control
BPA 10-10
BPA 10-8
BPA 10-6
C
EC50 = 0.15 nM
% increase in Ca2+ transient decline rate
BPA concentration (M)
D
BPA
Control
pPLN-T17 (CAMKII)
PLN total

Figure 3

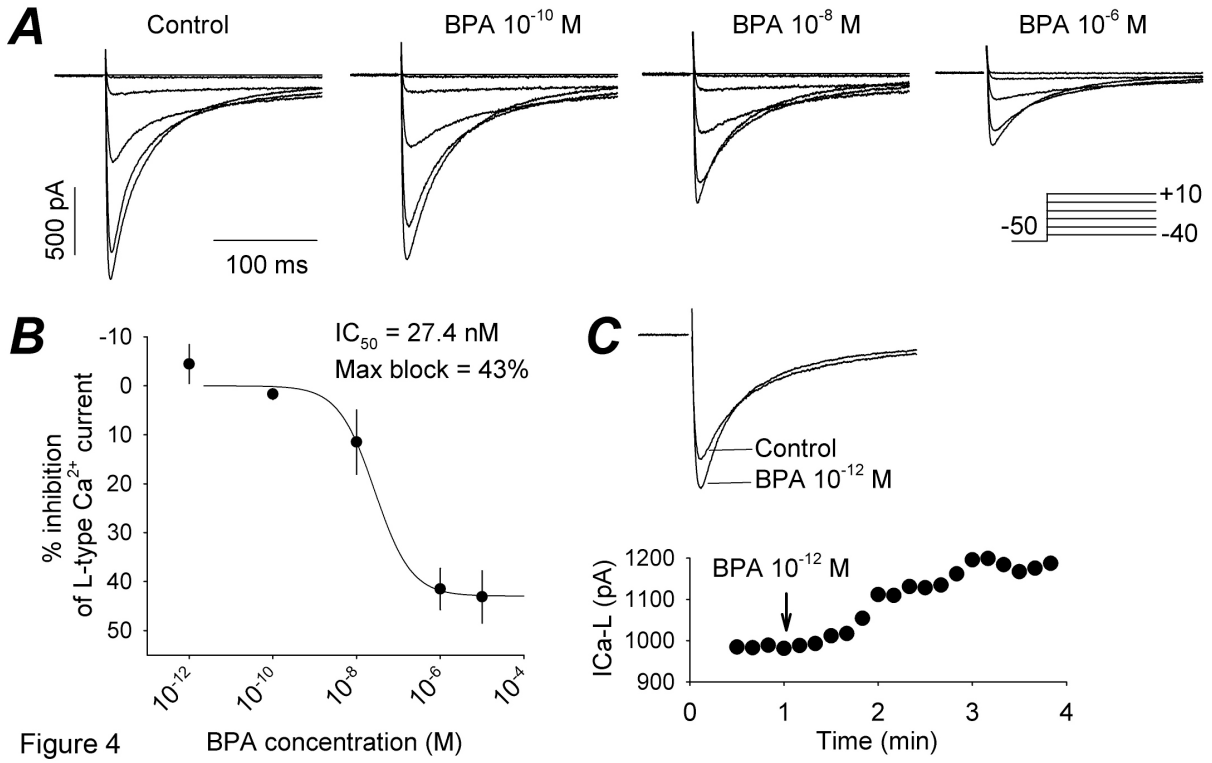

A
Control
BPA 10-10 M
BPA 10-8 M
BPA 10-6 M
500 pA
100 ms
+10
-50
-40
B
IC50 = 27.4 nM
Max block = 43%
% inhibition of L-type Ca2+ current
-10
0
10
20
30
40
50
10-12
10-10
10-8
10-6
10-4
BPA concentration (M)
C
Control
BPA 10-12 M
BPA 10-12 M
ICa-L (pA)
1200
1100
1000
900
0
1
2
3
4
Time (min)

Figure 4

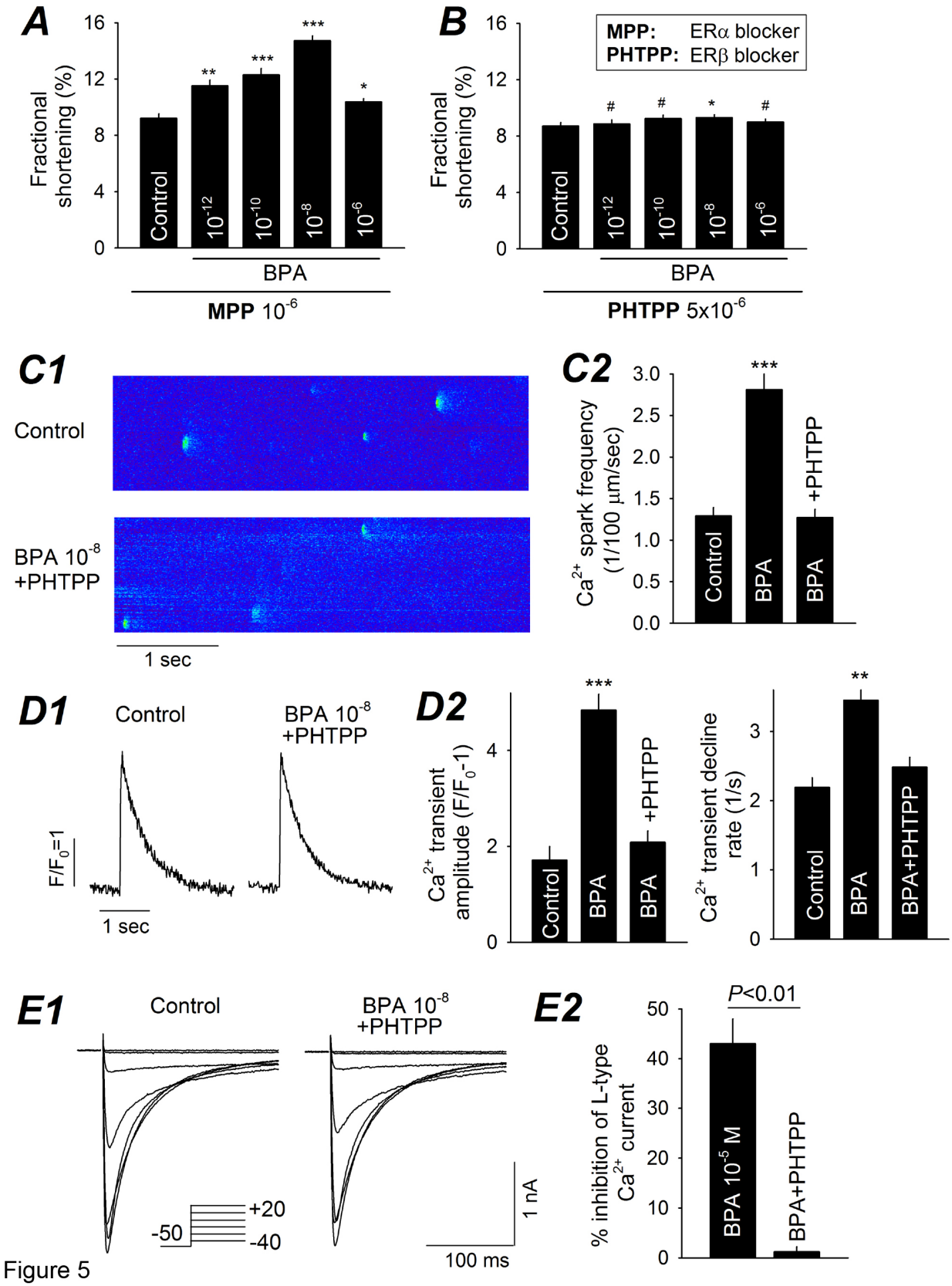

A
Fractional shortening (%)
16
12
8
4
0
Control
10-12
10-10
10-8
10-6
**
***
***
*
BPA
MPP 10-6
B
MPP: ERα blocker
PHTPP: ERβ blocker
#
#
*
#
PHTPP 5x10-6
C1
Control
BPA 10-8
+PHTPP
1 sec
C2
Ca2+ spark frequency (1/100 μm/sec)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
Control
BPA
BPA
+PHTPP
D1
Control
BPA 10-8
+PHTPP
F/F0=1
1 sec
D2
Ca2+ transient amplitude (F/F0-1)
4
2
0
***
Control
BPA
BPA
+PHTPP
Ca2+ transient decline rate (1/s)
3
2
1
0
**
Control
BPA
BPA+PHTPP
E1
Control
BPA 10-8
+PHTPP
+20
-50
-40
1 nA
100 ms
E2
P<0.01
% inhibition of L-type Ca2+ current
50
40
30
20
10
0
BPA 10-5 M
BPA+PHTPP

Figure 5

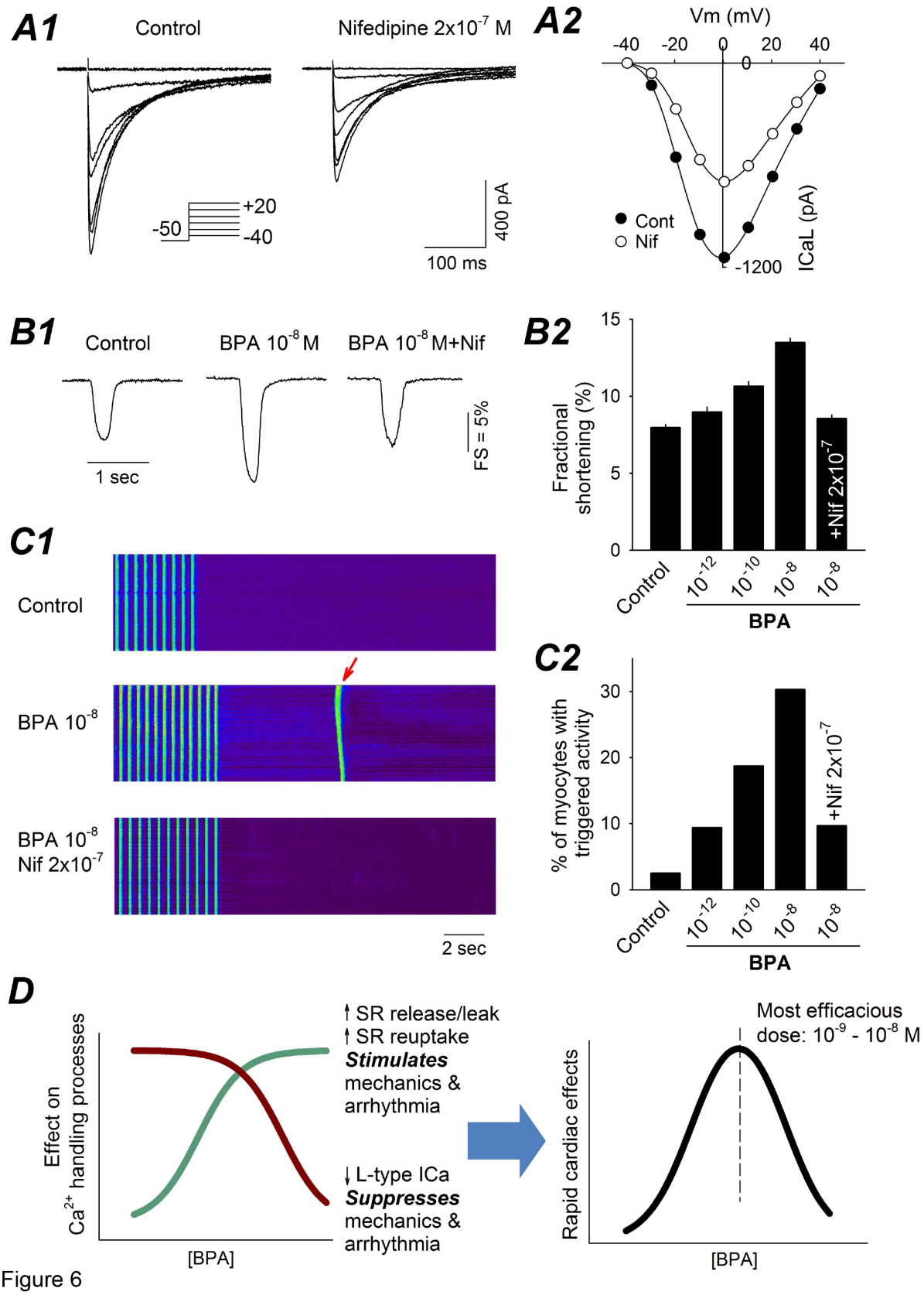

A1
Control
Nifedipine 2x10-7 M
+20
-50
-40
400 pA
100 ms
A2
Vm (mV)
-40
-20
0
20
40
0
Cont
Nif
-1200
ICaL (pA)
B1
Control
BPA 10-8 M
BPA 10-8 M+Nif
FS = 5%
1 sec
B2
Fractional shortening (%)
15
10
5
0
+Nif 2x10-7
Control
10-12
10-10
10-8
10-8
BPA
C1
Control
BPA 10-8
BPA 10-8
Nif 2x10-7
2 sec
C2
% of myocytes with triggered activity
30
20
10
0
+Nif 2x10-7
Control
10-12
10-10
10-8
10-8
BPA
D
Effect on Ca2+ handling processes
[BPA]
↑ SR release/leak
↑ SR reuptake
Stimulates mechanics & arrhythmia
↓ L-type ICa
Suppresses mechanics & arrhythmia
Most efficacious dose: 10-9 - 10-8 M
Rapid cardiac effects
[BPA]

Figure 6